Transcription-induced barriers to supercoil diffusion in the *Salmonella typhimurium* chromosome

Shuang Deng, Richard A. Stein, and N. Patrick Higgins*

Department of Biochemistry and Molecular Genetics, University of Alabama, Birmingham, AL 35294-2170

Edited by Susan Gottesman, National Institutes of Health, Bethesda, MD, and approved January 13, 2004 (received for review November 14, 2003)

Transcription and replication both influence and are influenced by superhelical changes in DNA. Explaining how supercoil movement is channeled in living chromosomes has been a major problem for 30 years. Transcription of membrane-associated proteins leads to localized hypersupercoiling of plasmid DNA, and this behavior indicates the presence of aberrant supercoil diffusion. Using the lambda Red recombination system, we constructed model domains in the *Salmonella typhimurium* chromosome to analyze supercoiling dynamics of regions encoding membrane proteins. Regulation of Tn*10*-derived tetracycline resistance involves a repressor, TetR, and a membrane-bound export pump, TetA. Strains deficient in TetR activity had 60-fold higher transcription levels (from P_A) than TetR-positive strains. High *tetA* transcription caused a 10- to 80-fold decrease in the γδ resolution efficiency for the domain that includes the Tet module. Replacing *tetA* with genes encoding cytosolic proteins LacZ and Kan also caused the appearance of supercoil diffusion barriers in a defined region of the chromosome. In strains containing a functional TetR located next to a regulated *lacZ* reporter (P_R*tetR*-P_A*lacZ*), induction of transcription with chlortetracycline caused a 5-fold drop in resolution efficiency in the test domain interval. A short half-life resolvase showed that barriers appeared and disappeared over a 10- to 20-min span. These studies demonstrate the importance of transcription in chromosome structure and the plasticity of supercoil domains in bacterial chromosomes.

gyrase | site-specific recombination | γδ resolvase | domains | ClpXP

Numerous studies demonstrate that bacterial chromosomes are partitioned into independent domains that limit supercoil diffusion (1, 2). Early analyses of bacterial chromosomes liberated from cells by treatment with lysozyme and detergent showed that "nucleoids" were negatively supercoiled objects and that multiple single-strand breaks were needed to relax all supercoils (3, 4). Sinden and Pettijohn subsequently confirmed that chromosomes were segregated into multiple independent domains *in vivo* by using tri-methylpsoralen crosslinking (5). In 1996, an assay was developed to measure supercoil diffusion inside living cells by using the site-specific recombination reaction of Tn*3* or the closely related element γδ (6). In this assay, the ability to delete specific chromosome segments separated by two γδ *res* sites spaced along the chromosome measures supercoil diffusion (7, 8). What elements are responsible for domain structure is a long-standing question.

One advantage of the site-specific resolution assay is that it shows how supercoil structure at specified locations is modified when growth rate or biological activity changes. Most resolution barriers are random with respect to DNA sequence, but the appearance of a new barrier has been documented. For example, induction of the early promoter of the bacteriophage Mu caused a new barrier to supercoil diffusion that was restricted to the region near the virus center (9).

The impact of transcription on DNA structure is well documented in plasmids (10). For example, transcription of the pBR322 *tetA* gene causes dramatic hypersupercoiling when the cell also harbors a mutation in the *topA* gene, which encodes a type I topoisomerase (ω protein) (11–14). Studies by Lynch and Wang showed that hypersupercoiling from a moderate-strength promoter required both the presence of a *topA* mutation plus transcription of a protein that was cotranscriptionally inserted into the membrane or exported to the periplasm or outer membrane (15). Similar studies have been difficult to conduct in the bacterial chromosome for two reasons. First, it has been difficult to construct test intervals in the 4-Mb bacterial genome. Second, bacterial chromosomes have supercoiling domains larger than most multicopy plasmids.

A new supercoil diffusion barrier appears after induction of *tetA* transcription. Moreover, transcription of two cytosolic proteins, LacZ and Kan, showed similar effects. A short half-life resolvase revealed that barriers appear and disappear over a 10- to 20-min span. These studies demonstrate an extremely dynamic domain structure for bacterial chromosomes.

Materials and Methods

Media. LB and minimal media were prepared as described (6). Antibiotics were added to medium at concentrations of 50 μg/ml for kanamycin and ampicillin, 20 μg/ml for chloramphenicol, 12 μg/ml for tetracycline, and 10 μg/ml for gentamicin. Bochner plates were made as described (16).

Plasmids. Plasmid pJBRES*c*I is a pACYC184-derived vector carrying the *tnpR* (WT Res resolvase protein) gene of transposon γδ cloned under control of the λP_L promoter with a nearby copy of λ *c*Its repressor gene (17). Plasmid pJBRES*c*I-SSRA carries a short half-life Res resolvase in which the sequence tag encoded by *ssrA* RNA was added to the carboxyl terminus of Res. λ Red helper plasmid pKD20 is a derivative of pINT-ts that encodes Red recombinase under the control of the P_{araB} promoter (18).

Chromosomal Modification with Red Recombination. Insertion mutagenesis of the *Salmonella typhimurium* chromosome was done with the lambda Red recombination system of plasmid pKD20 (18). Synthetic oligos used for each construct is described in Table 2, which is published as supporting information on the PNAS web site. Oligonucleotides were purchased from IDT (Coralville, IA). *Taq* polymerase (Sigma) and *Taq* extender (Stratagene) were mixed 1:1 to generate DNA for sequence analysis and to make gene-replacement substrates. Recombinants with the correct insertions were selected for tetracycline resistance or kanamycin resistance or screened for blue-color phenotype and tested as described (6, 19). Details of strain constructions are given in *Supporting Text*, which is published as supporting information on the PNAS web site.

Assays. To measure resolution, log-phase cultures grown at 30°C to 50 Klett units (filter no. 54) were induced for Res expression by a 10-min incubation at 42°C followed by 10-fold dilution in LB containing chloramphenicol. Cells were incubated overnight at

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: CLT, chlortetracycline hydrochloride; β-gal, β-galactosidase.

*To whom correspondence should be addressed. E-mail: nphiggins@bmg125.cmc.uab.edu.

www.pnas.org/cgi/doi/10.1073/pnas.0307550101

Table 1. Strains used

Strain	Genotype	Plasmid
NH3470	*zbj8904*::Tn*10d*(Gn)◇(*kan*) (*ylil*)◇(P_R*tetR435-*P_A*tetA-res*)	pJBREScI
NH3471	*zbj8904*::Tn*10d*Gn (*ylil*)◇(*kan-res*)	pJBREScI
NH3472	*zbj8904*::Tn*10d*(Gn)◇(*kan*) (*ylil*)◇(P_R*tetR-*P_A*tetA-res*)	pJBREScI
NH3475	*zbj8904*::Tn*10d*(Gn)◇(*kan*) (*ylil*)◇(P_A*tetA-res*)	pJBREScI
NH3477	(*ylil*)◇(P_R*tetR435-*P_A*lacZ-res*)	
NH3478	(*ylil*)◇(P_R*tetR-*P_A*lacZ-res*)	
NH3479	(*ylil*)◇(P_A*lacZ-res*)	
NH3480	*zbj8904*::Tn*10dkan* (*ylil*)◇(P_A*tetA-rrnB-res*)	pJBREScI
NH3481	*cobP712*::Tn*10d*Gn *cobT714*::MudJr2	pJBREScI
NH3482	*cob-708*::Tn*10d*Gn *cobT714*::MudJr2	pJBREScI
NH3483	*cobT712*::Tn*10d*Gn *cobT714*::MudJr2(*kan*)◇(P_A*tetA*)	pJBREScI
NH3484	*cobP712*::Tn*10d*Gn *cobT714*::MudJr2(*kan*)◇(P_A*tetA*$_{inv}$)	pJBREScI
NH3485	*cob-708*::Tn*10d*Gn *cobT714*::MudJr2(*kan*)◇(P_A*tetA*)	pJBREScI
NH3486	*cob-708*::Tn*10d*Gn *cobT714*::MudJr2(*kan*)◇(P_A*tetA*$_{inv}$)	pJBREScI
NH3487	*zbj8904*::Tn*10d*(Gn)◇(*kan*) (*ylil*)◇(P_R*tetR-*P_A*lacZ-rrnB-res*)	pJBREScI
NH3488	*zbj8904*::Tn*10d*(Gn)◇(*kan*) (*ylil*)◇(P_A*lacZ-rrnB-res*)	pJBREScI
NH3489	*zbj8904*::Tn*10d*Gn (*ylil*)◇(P_R*tetR-*P_A*kan-rrnB-res*)	pJBREScI
NH3490	*zbj8904*::Tn*10d*Gn (*ylil*)◇(P_A*kan-rrnB-res*)	pJBREScI
NH3491	(*recN*)◇(P_R*tetR-*P_A*tetA*) *zbj8904*::Tn*10d*(Gn)◇(*kan*) (*ylil*)◇(P_A*lacZ-rrnB-res*)	pJBREScI
NH3492	*zbj8904*::Tn*10d*(Gn)◇(*kan*) (*ylil*)◇(P_R*tetR-*P_A*lacZ-rrnB-res*)	pJBREScI-SSRA

All strains were derived from *S. typhimurium* LT2 and were constructed during this work. The Tn*10d*Gn and MudJr2 elements were described (6). ◇ indicates a replacement generated by lambda Red recombineering (47).

30°C and then diluted 1:10^6 and plated on minimal medium with chloramphenicol and 5-bromo-4-chloro-3-indolyl β-D-galactoside. Deletion frequencies were scored by loss of color or loss of antibiotic resistance (6). To measure *lacZ* expression, overnight bacterial cultures diluted 1:100 in 5 ml of LB were grown to 50 Klett units. β-Galactosidase (β-gal) assays were done in triplicate as described (20).

Results

Supercoil Diffusion in the 17-Min Region of *S. typhimurium*. Transcription can lead to anomalous DNA structure and a hypernegative supercoiling phenotype in plasmid DNA (14, 15, 21, 22). For efficient promoters like the hybrid *tac* promoter under control of the Lac repressor, transcriptional effects on plasmid DNA topology can be documented in WT cells (23). With weaker promoters like the one regulating the tetracycline resistance gene on pBR322, cotranscriptional translation of membrane proteins stimulate hypernegative supercoiling only in *topA* mutants (15, 24). Although several examples of translation-driven supercoiling are restricted to *topA* mutants, the behavior has led to theories of cotranscription membrane association, helping to organize chromosomal DNA after replication (25, 26). A serendipitous result suggested that the *tetA* gene of Tn*10* might also cause topological anomalies in the chromosome. A genetic interval in *S. typhimurium* was flanked with a pair of *res* sites by using two combinations of drug markers. Strain NH3470 had a Tn*5* kanamycin resistance gene (*kan*) upstream and a Tn*10*-derived *tetR*/*tetA* module downstream. Strain NH3471 had a Tn*1696*-derived gentamicin resistance gene upstream (*gen*) and the Tn*5* *kan* gene downstream (Table 1 and Fig. 1*A*). Recombination between *res* sites in strain NH3470 (Fig. 1*A Upper*) results in sensitivity to tetracycline, and in NH3471 (Fig. 1*A Lower*) resolution leads to kanamycin sensitivity.

Based on studies of the genetic segment between the *cob* and *his* operons, we expected resolution efficiency for a 12-kb interval to be 80% (Fig. 1*B*, first column). However, NH3470 had a resolution efficiency of only 3%, which is 26-fold lower than expected (see Fig. 1*B*, second column). Strain NH3471, analyzed by using the same protocol except that kanamycin sensitivity was scored, had a resolution frequency of 57%, which is close to the expected level (Fig. 1*B*, third column). This unexpected low efficiency suggested that the *tet* cassette in strain NH3470 caused a low-resolution phenotype.

We have used Tn*10*-derived *tet-res* elements for resolution analyses in many different intervals where they did not show a low-resolution phenotype. One explanation is that a mutation (like an altered *res* site) generated during PCR amplification reduced resolution efficiency. To test this hypothesis, a new strain was made by using different PCR reactions to generate the *tet-res* module. A resulting strain (NH3472) had a resolution efficiency of 56%, which was similar to the efficiency of the strain NH3471 (57%). This experiment supports the notion that the *tet* module in strain NH3470 was unusual.

Sequence analysis of *tet* modules from NH3470 and NH3472 showed that NH3470 *res* site was unaltered, but it had a deletion of base pair 435 in the *tetR* gene (ΔA-435). Hereafter, we refer to this allele as *tetR435.* The deletion causes a frameshift and premature translation stop that eliminates 66 amino acids of the WT TetR protein (Fig. 6, which is published as supporting information on the PNAS web site). Although the DNA-binding domain of TetR435 repressor is unaltered (27), destabilization of the TetR dimer interface might lead to constitutive *tetA* gene expression (28).

Tet expression in strains with WT *tetR* and *tetR435* alleles was measured in two ways. First, Bochner plates were used to test sensitivity to fusaric acid. These plates are commonly used to counterselect against strains carrying Tn*10*-derived tetracycline resistance (16). TetA expression is toxic at 42°C in the presence of fusaric acid. Standard Bochner plates contain chlortetracycline hydrochloride (CLT), which induces the Tet operon by binding the TetR, causing its dissociation from DNA and transcription of both *tetA* and *tetR* (28). Fusaric acid sensitivity was measured in normal Bochner plates and modified plates lacking CLT. Strain NH3472 with a WT *tetR* gene grew well on Bochner plates without CLT but was killed on a standard Bochner plate (Fig. 7, which is published as supporting information on the PNAS web site). Strain NH3470 died on Bochner plates with or without CLT, which indicates constitutive TetA expression.

Second, to quantify transcription from the *tetA* promoter,

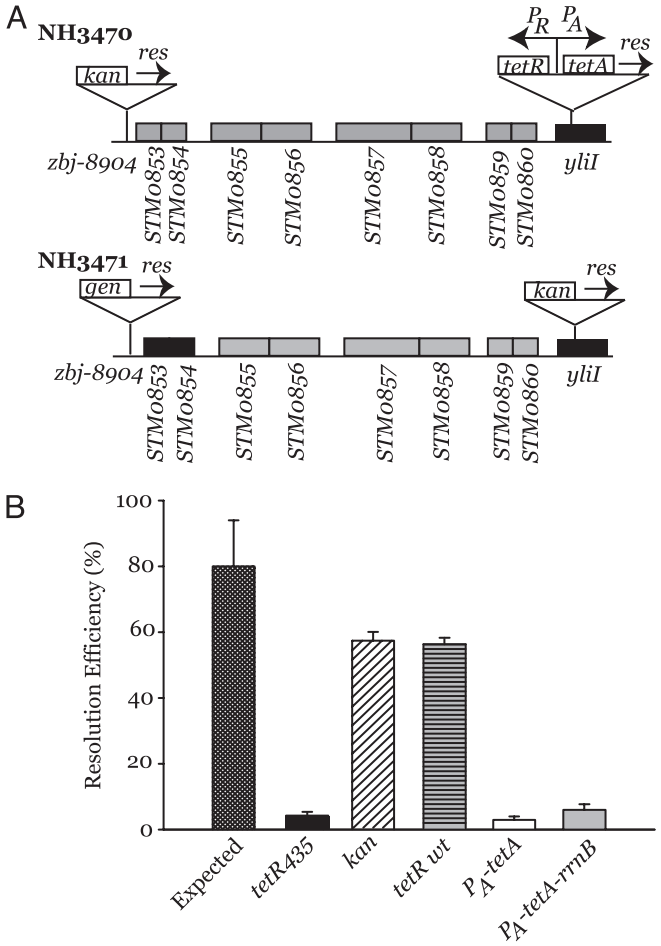

Fig. 1. Effect of WT *tetR*, a mutant *tetR*, and a *tetR* deletion on γδ resolution efficiency. (*A*) Physical and genetic map of a 12-kb interval between *zbj8904* and *yliI* in the 17- to 20-min region of *S. typhimurium* includes eight ORFs flanked by drug markers and *res* sites. Two arrangements of selectable drug markers involve kanamycin and tetracycline resistance (*Upper*) and gentamicin and kanamycin resistance (*Lower*). (*B*) Resolution efficiency comparison among different module insertions in the gene *yliI* in the 12-kb interval in the log phase. Resolution efficiency for a 12-kb interval in the 43- to 45-min region (6) is designated as the expected resolution efficiency.

chromosomal *lacZ* was fused to a *tetA* promoter (P_A) by using lambda Red substitution. NH3478 (WT *tetR*) made 15 units of β-gal in the absence of CLT and 688 units of β-gal after addition of 5 μg/ml CLT. NH3477 (*tetR435*) made 959 units of β-gal with or without CLT addition (Table 3, which is published as supporting information on the PNAS web site). Thus, *tetR435* had a higher level of *tetA* expression than a WT *tetR* construct induced with the optimal amount of CLT. The effect of *tetA* expression was local and not generally inhibitory to resolution, because a Tet element 1,800 kb away from the test interval had no impact when the region had no *tetA* promoter (data not shown).

Transcription Hinders Supercoil Diffusion. Although the expression data shown above reveal a correlation between *tetA* expression and reduced γδ resolution, transcription and translation levels of TetA are not the only differences between these strains. The TetR435 protein might cause DNA damage or alter DNA structure by unexpected mechanisms. To check this possibility, strains NH3475 and NH3479 were made, which lack TetR and constitutively express *tetA* or *lacZ*, respectively, from the P_A promoter. NH3475 (P_A*tetA* allele) was similar to NH3470, being inviable on Bochner plates with or without CLT (Fig. 7). Strain NH3479 was similar to NH3477 because it expressed 915 units of β-gal (Table 3). The resolution efficiency of a strain lacking TetR (NH3475) (*yliI*)<>(P_A*tetA-res*) was identical (3%) with the strain carrying the *tetR435* allele (Fig. 1*B*, fifth column). Thus, *tetR435* and a *tetR* deletion have the same phenotype.

The hypothesis at this point was that TetA protein expression induces a position-specific barrier to chromosome supercoil diffusion, perhaps by handcuffing the DNA to the cell membrane during translation. However, a trivial explanation is that RNA polymerase transcribes through the *res* site, causing Res protein to dissociate. This mechanism would block site-specific resolution by occluding Res binding to one *res* site. To address this possibility, a pair of efficient transcription terminators (*rrnB* T/T2) was placed at the 3′ end of *tetA*. After the TetA termination codon is a 105-bp sequence followed by the *rrnB* T1/T2 terminators (158 bp in length), and the γδ *res* site is 129 bp beyond the terminators. In this configuration the *res* site is shielded from occlusion by efficient (>95%) transcription terminators. Strain NH3480, which contains this construct (*yliI*)<>(P_A*tetA-rrnB-res*), retained a low-resolution phenotype (6% recombinants; see Fig. 1*B*, sixth column). A 2-fold occlusion effect is possible, because the resolution efficiency rose from 3% without the terminator to 6% when the terminator was present. Nonetheless, most of the inhibitory effect of *tetA* expression on resolution remained to be explained.

TetA Barriers Are Mobile. To see whether *tetA* expression alters supercoiling behavior in a different chromosomal setting, several *tetA* configurations were moved to the *his-cob* region. This genetic interval has been thoroughly characterized, and it has no sequence- or region-specific barriers (6, 19, 29). Two intervals that normally resolve at high frequency include a 14-kb segment defined by *cobP712*::Tn*10d*Gn and *cobT714*::Mu*d*Jr2 (Fig. 2*A*, NH3481) and a 28-kb interval defined by *cob-708*::Tn*10d*Gn and *cobT714*::Mu*d*Jr2 (Fig. 2*A*, NH3482). In these experiments, a Δ*tetR* P_A*tetA* module was substituted for the *kan* gene in *cobT714*::Mu*d*Jr2 (Fig. 2*B*). Replacements were made in two orientations to control for transcription occlusion. In NH3483 and NH3485, *tetA* transcription points toward the nearby *res* site [Fig. 2*B*, Mu*d*Jr2 (*kan*)<>(P_A*tetA*)]. In NH3484 and NH3486 *tetA* transcription is directed away from the *res* site [Fig. 2*B*, Mu*d*Jr2 (*kan*)<>(P_A*tetA-inv*)]. In this orientation RNA polymerase cannot occlude a *res* site.

Resolution efficiency over the 14-kb interval between *cobP712*::Tn*10d*Gn and *cobT714*::Mu*d*Jr2 (NH3481) was 98% (Fig. 2*Ca*, Mu*d*Jr2). When the *kan* gene in Mu*d*Jr2 was replaced by P_A*tetA* (NH3483), resolution efficiency dropped to 2% (Fig. 2*Ca*, P_A*tetA*). When the *kan* gene was replaced by inverted P_A*tetA* (NH3484), resolution efficiency was 5% (Fig. 2*Ca*, P_A*tetA-inv*). Thus, constitutive *tetA* expression caused a 20- to 50-fold decrease in resolution efficiency for this 14-kb interval. Resolution efficiency for the 28-kb interval between *cob-708*::Tn*10d*Gn and *cobT714*::Mu*d*Jr2 (NH3482) was 78% (Fig. 2*Cb*, Mu*d*Jr2). Replacement of the *kan* gene in Mu*d*Jr2 with P_A*tetA* (NH3485) caused resolution efficiency to decrease to 1% (Fig. 2*Cb*, P_A*tetA*). When *kan* gene was replaced by an inverted P_A*tetA* (NH3486), resolution efficiency was 2% (Fig. 2*Cb*, P_A*tetA-inv*). Constitutive *tetA* expression reduced resolution efficiency in the 28-kb interval by 40- to 80-fold. These results prove that the *tetA* gene is a mobile element that can disrupt supercoil diffusion. Occlusion here is unlikely in either case, so the major impact is due to transcription and/or translation of *tetA*.

Genes Encoding Cytoplasmic Proteins Also Create Supercoil Barriers. Is membrane association necessary to disrupt supercoil diffusion? To address this question, we measured γδ resolution in intervals with *lacZ* and *kan* genes fused to the *tetA* promoter.

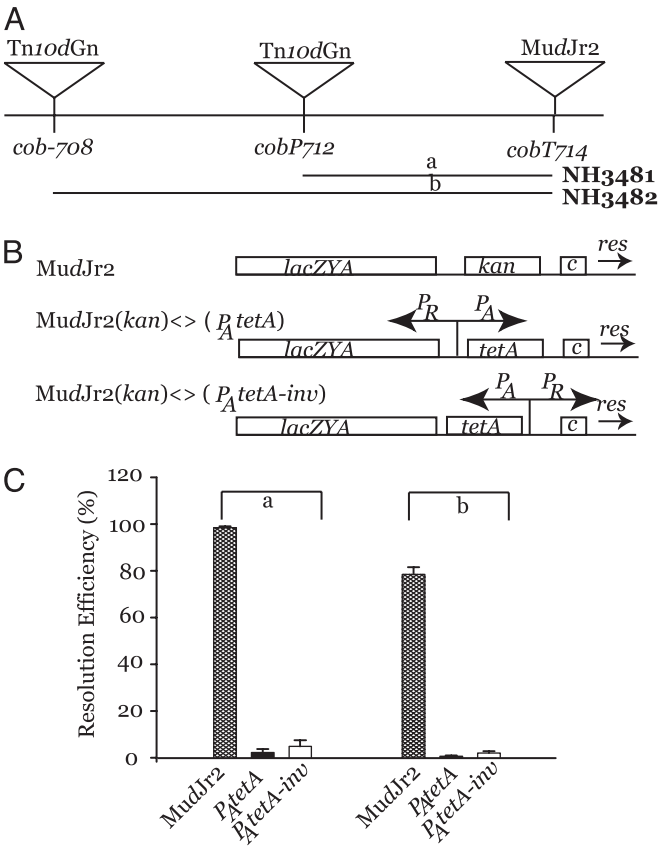

Fig. 2. γδ resolution in the 43- to 45-min of *S. typhimurium* chromosome. (*A*) Physical map of two intervals. The 14-kb interval from *cobP712* to *cobT714* is labeled a, and the 28-kb interval from *cob-708* and *cobT714* is labeled b. (*B*) Genetic map of *res* elements placed at the *cobT714* location: MudJr2, MudJr2(*kan*)<>(P_A*tetA*), and MudJr2(*kan*)<>(P_A*tetA-inv*). (*C*) Resolution efficiencies for different 14-kb (*a*) and 28-kb (*b*) intervals.

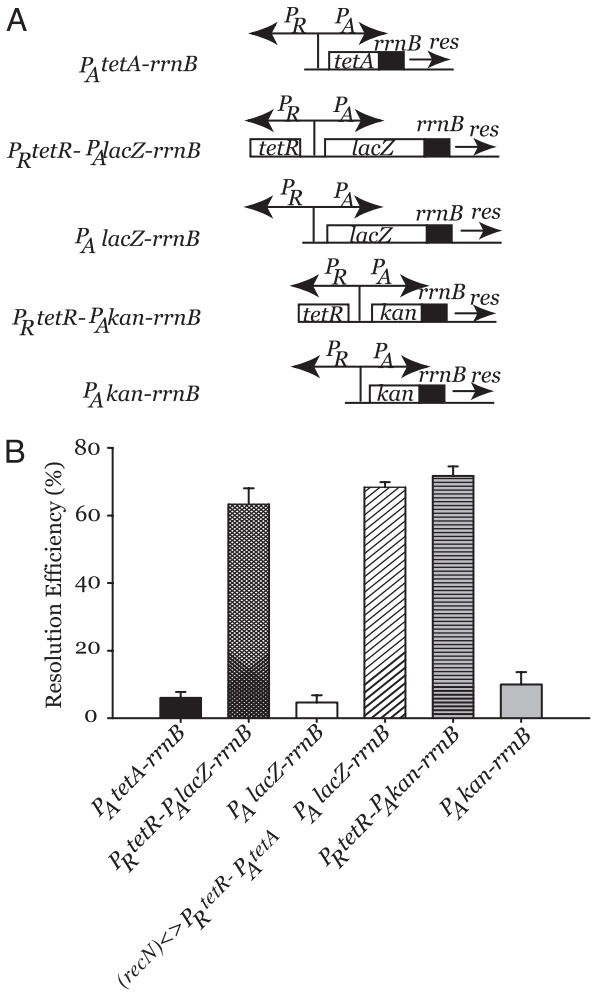

Fig. 3. Effect of genes encoding cytoplasmic proteins on the γδ resolution efficiency in the 12-kb interval between *zbj8904* and *ylil*. (*A*) Genetic map of different protein coding sequences (*tetA*, *lacZ*, and *kan*) fused to *tetA* promoter P_A. (*B*) Resolution efficiency comparison among different insertions in the gene *ylil* in the 12-kb interval in the log phase.

When *lacZ* expression was repressed by TetR (NH3487), γδ resolution was efficient (65% in Fig. 3 *A* and *B*, column 2), but when *lacZ* expression was unregulated (NH3488), γδ resolution was very inefficient (4% in Fig. 3 *A* and *B*, column 3). The magnitude of disruption to supercoil diffusion in NH3488 was similar to a strain constitutive for TetA expression (6% in Fig. 3 *A* and *B*, column 1). Similarly, when *kan* was placed at P_A under the control of TetR (NH3489), γδ resolution was efficient (70% in Fig. 3 *A* and *B*, column 5). But unregulated *kan* expression (NH3490) caused γδ resolution to fall to the level of cells expressing TetA (7% in Fig. 3 *A* and *B*, column 6). Clearly, a membrane anchor is not a prerequisite for generating barriers to supercoil diffusion; transcription of two genes encoding cytoplasmic proteins reduced γδ resolution as much as transcription of *tetA*.

To see whether resolution could be modulated by using a distant source of *tetR* repressor, *tetR* was cloned into *recN* (NH3491), which is 1,800 kb away from gene *yliI.* With no source of TetR, strain NH3488 made dark-blue colonies, and resolution was an inefficient 4% (Fig. 3 *A* and *B*, column 3). When TetR protein was provided in trans, NH3491 made light-blue colonies and γδ resolution was efficient (67%, Fig. 3 *A* and *B*, column 4). Thus, TetR regulation of the *tetA* promoter clearly determines the efficiency of resolution reactions.

Barrier Kinetics. Supercoil barriers in two regions of the genome appear when genes encoding *tetA, lacZ*, or *kan* are expressed from a *tetA* promoter P_A (Figs. 1–3). To explore the correlation between transcription level and γδ resolution efficiency, a dose–response experiment was carried out in strain NH3492, which has the *lacZ* fused to the *tetA* promoter next to a WT *tetR* repressor. Cultures grown overnight in LB were diluted 100-fold in medium containing 0, 0.1, 0.5, 1, 5, 10, or 20 μg/ml CLT. Cell growth at 30°C was monitored in Nephlo flasks by using a Klett meter, and when cultures reached the value of 50 Klett units, half of the cells were harvested by centrifugation and assayed for β-gal activity. The remaining half of each culture was incubated at 42°C for 10 min to induce expression of a tagged form of γδ Res protein (see below). Cells were put back in culture at 30°C to allow chromosome segregation and then diluted and spread on plates containing the chromogenic indicator 5-bromo-4-chloro-3-indolyl β-D-galactoside. Resolution efficiency was calculated as the percentage of white colonies among all colonies. WT Res protein is stable, and once induced, it can catalyze recombination for a time lasting longer than two cell divisions, which obscures the kinetic analysis of barrier disappearance. The Res-SsrA protein has a C-terminal extension of 11 amino acid residues that are identical with the Clp-XP proteolysis tag of the SsrA system (30). Research (R.S., unpublished work) shows that this protein has a half-life of 5 min in exponential cultures of *S. typhimurium*. The Res-SsrA protein is fully functional and has

BIOCHEMISTRY

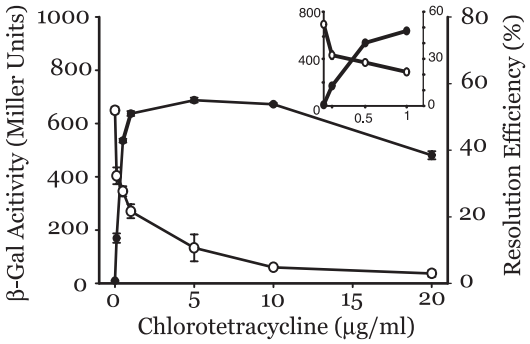

Fig. 4. Effect of chlortetracycline concentration on β-gal activity and γδ resolution efficiency. NH3492 was subcultured in 0, 0.1, 0.5, 1, 5, 10, and 20 μg/ml chlortetracycline and was grown to a cell density of 50 Klett units. Both β-gal assays (●) and γδ assays (○) were performed on each sample.

the same *in vivo* resolution efficiency as WT Res if either the expressing cells lack functional ClpXP protease or the 11-aa tag is modified so that it is no longer recognized as a ClpXP/P substrate (data not shown). For a 10-min induction followed by outgrowth at 30°C, 90% of all resolution in strains with the Res-SsrA protein occurs in a 5- to 12-min window after the shift to 42°C.

A reciprocal relationship between β-gal activity and γδ resolution efficiency was observed (Fig. 4). At 0.1 μg/ml CLT, a small induction of β-gal expression produced a small inhibitory effect on resolution (Fig. 4 *Inset*). With the increase of CLT concentration, β-gal expression increased and resolution decreased. At 5 μg/ml CLT, β-gal activity peaked and γδ resolution efficiency approached a minimum. At very high concentrations of CLT, cells grow poorly, which complicates both resolution and β-gal assays.

To study the kinetics of barrier appearance, fresh overnight cultures of NH3492 were diluted 100-fold into LB at 30°C, and cell growth was followed in a Klett meter. At a density of 50 Klett units, 5 μg/ml CLT was added. One aliquot was immediately induced for the resolution assay, then at various later time points aliquots were removed and shifted to 42°C for resolution assays (Fig. 5). Each time point in Fig. 5 marks the elapsed time from addition of CLT to the end of the 42°C induction period.

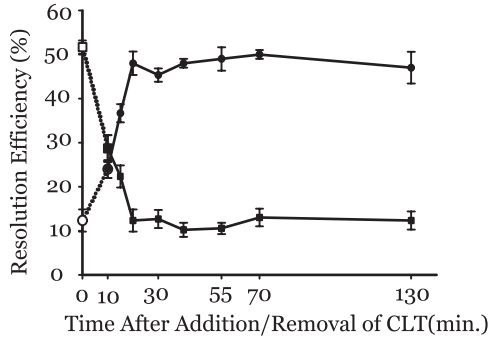

Fig. 5. Barrier appearance and disappearance in response to chlortetracycline. □, overnight cultures of NH3492 subcultured in LB without chlortetracycline. γδ resolution assays were done when cell density reached 50 Klett units. ■, 5 μg/ml chlortetracycline added at a cell density of 50 Klett units. Samples were taken at various points after addition of chlortetracycline and subjected to γδ resolution assay. ○, NH3492 grown in LB with 5 μg/ml chlortetracycline. γδ resolution assays were initiated when cell density reached 50 Klett units. ●, chlortetracycline removed by quickly washing and then resuspending cells in LB. Samples taken at various times were tested for γδ resolution.

Resolution frequencies changed 5-fold from 50% to 10% during a 20-min window of growth and induction with CLT.

The kinetics of barrier disappearance was also examined. An overnight culture of NH3492 was diluted 100-fold into LB plus 5 μg/ml CLT. At a density of 50 Klett units, cells were harvested and resuspended in CLT-free medium, and 10-min shifts to 42°C were done in the same manner described above. Resolution efficiency increased from 10% to 50% after CLT removal. Barriers appeared and disappeared with similar kinetics (Fig. 5).

Discussion

Transcription changes the dynamic behavior of plasmid DNA in several ways. First, RNA polymerase unwinds the double helix as it transcribes RNA. Induction of a moderate-strength promoter can add six new RNA polymerase molecules to a small plasmid, which changes the average linking number by 10 negative supercoils (31). Second, transcription causes a temporary segregation of positive supercoils in front of a transcribed gene and negative supercoils in the transcription wake (32–34). In strains carrying a *topA* mutation, expression of a membrane-inserted protein can result in a dramatic increase in negative supercoil of plasmids (14, 15, 21). Both of these effects are visible because rapid plasmid extraction freezes the population in its current topological state, and agarose gel electrophoresis can detect subtle changes in plasmid topology (35). Two effects of transcription have been noted in the large bacterial chromosome. First, if a highly transcribed operon like *rrnB*, which encodes ribosomal RNAs, is positioned head to head with a replication fork, transcription markedly delays the time required for forks to cross the transcription unit (36). Second, induction of the strong phage Mu early promoter causes a new supercoil barrier to appear in the vicinity of the transcription unit (9).

In this work we found three effects of transcription on supercoil dynamics in the *S. typhimurium* genome. First, constitutive *tetA* transcription caused a persistent 20-fold reduction in γδ site-specific recombination over a 14-kb interval by (Fig. 2). The effect was larger for longer intervals, with an impact of a 40-fold reduction for a 28-kb domain. Second, transcription effects were not restricted membrane proteins. Similar effects on γδ resolution efficiency were found with aminoglycoside-3′-*O*-phosphotransferase, which is the product of the Tn*5 kan* gene and β-gal, the product of *Escherichia coli lacZ*. Barriers were detected by using a WT resolvase in combination with modules that either lacked a functional copy of the TetR repressor, or modules with WT TetR protein but in the presence of the inducer CLT. The critical determinant was transcription strength. Even though the eight genes separating the *res* sites in strain NH3470 include five membrane-associated proteins, the level of transcription of these genes, estimated by microarray analysis of total-cell RNA (data not shown), was too low to disrupt supercoil diffusion. At the *atp* operon, consisting of nine genes encoding membrane-associated proteins, and the *nmpc* operon, which regulates a single gene, the mRNA abundance measured from microarray analysis accurately predicted the impact on γδ resolution (Fig. 8, which is published as supporting information on the PNAS web site). Third, by using a modified form of γδ resolvase with a cellular half-life of 5 min, barriers to supercoil diffusion appeared and disappeared within 10–20 min of the point when CLT was either added or washed out of cell cultures. The impact of transcription over a 14-kb interval was correlated with the level of transcription over a 40-fold range of expression measured with β-gal assays.

Because transcription alters chromosome dynamics of both plasmid and the bacterial chromosomal DNA, the question arises as to how many transcription-related barriers exist in a bacterial cell. Microarray analyses provide important information on genome-wide transcription profiles (37, 38). Cells growing under the same conditions used in our experiments (exponential

cultures in LB) give detectable RNA signals for >4,000 *E. coli* genes (38). However, steady-state mRNA abundance exhibits a strikingly skewed distribution. For 70% of the genes (3,034 ORFs) mRNA abundance is less than one molecule per cell. Genes in this category include the uninduced RNA for *lacZ* (which yields ≈15 Miller units of β-gal activity) and 69 of the 321 known essential genes. Genes in this low-transcription category probably have little impact on DNA dynamics. The second class is genes with a steady-state abundance of one to four copies of RNA/DNA, which results in 40–160 Miller units of β-gal activity. Genes in this class (≈1,000) include 86 essential genes, and they are also predicted to have a modest topological impact. Fig. 4 suggests that over a 14-kb interval these genes would decrease resolution by 30% or less. The third gene class includes genes with RNA/DNA ratios >4. Included in this category would be a *lacZ* gene induced with 1 mM isopropyl β-D-thiogalactoside, which causes a 40- to 60-fold derepression of transcription to ≈600 Miller units. Protein-coding genes in *E. coli* with RNA/DNA ratios of 10 or greater represent 85 genes. The predicted impact on chromosome domains of these top 85 genes is given in Table 4, which is published as supporting information on the PNAS web site. The abundance of *lacZ* mRNA in uninduced cultures yields an RNA/DNA ratio of 0.45 (38), so a culture with 600 Miller units would have an RNA/DNA ratio of ≈20. This expression change is nearly identical with the change in levels of β-gal expression we observe in *S. typhimurium* going from a repressed P_R*tetR*-P_A*lacZ* culture to steady-state expression in the presence of 5 μg/ml CLT (Fig. 4). Extrapolating from the plot in Fig. 4, the number of chromosomal sites with diffusion barriers that would inhibit resolution by 5-fold or more would be <8. If we add the seven ribosomal operons, we predict that only ≈15 sites would show persistent 5-fold effects on resolution. This number of barriers is small compared with our estimate of 300 barriers that are stochastic and associated with DNA replication.

Several implications of these results are noteworthy. First, because transcription of many genes, including a significant fraction of the essential genes, is intermittent in many parts of the chromosome, domain structure is likely to be both dynamic and variable. Recent studies show that cell-to-cell expression variation is high for the *lac* operon at low inducer levels (39, 40). In specific instances, a burst of transcription can lead to a large amount of protein production in some cells and none at all in most cells. Considering the fact that operons like *araBAD* respond in similar fashion (41), no two organisms in a bacterial culture may ever have the same genome organization. This dissimilarity makes chromosome structure behave like a quasi-species, which is a collection of organisms that vary in structure around a common mean (42). Second, the genome behavior of the highly expressed operons may have special mechanisms to insulate their transcriptional effects from surrounding chromosome regions. The highly transcribed genes may be under selection for interspersion. Examples are known where chromosomal inversions involving specific inverting sites are disfavored, whereas other inversions using the same end points are permitted (43, 44). Finding the rules governing such behavior has been difficult. It is possible that apposition of two chromosomal segments with high-transcription activity may be unstable, just as placing a replication origin in front of a strong transcription unit leads to an impediment to replication (36). Third, recent reports in both bacteria and yeast show that high levels of transcription can cause formation of persistent RNA:DNA hybrids (R-loops) that interfere with both transcription and replication (22, 45, 46). Whether R-loop formation is responsible for some examples of chromosomal domain behavior is a difficult question, but it is one well worth pursuing.

We thank Dipankar Manna, Keith Champion, and Zhenhua Pang for critical comments on the manuscript. This work was supported by National Institutes of Health Grant GM33143-15.

1. Pettijohn, D. E. (1996) in *Escherichia coli and Salmonella*, ed. Neidhardt, F. C. (Am. Soc. Microbiol., Washington, D.C.), Vol. 1, pp. 158–166.
2. Higgins, N. P. (1999) in *Organization of the Prokaryotic Genome*, ed. Charlebois, R. L. (Am. Soc. Microbiol., Washington, D.C.), Vol. 1, pp. 189–202.
3. Stonington, G. O. & Pettijohn, D. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 6–9.
4. Worcel, A. & Burgi, E. (1972) *J. Mol. Biol.* **71,** 127–147.
5. Sinden, R. R. & Pettijohn, D. E. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 224–228.
6. Higgins, N. P., Yang, X., Fu, Q. & Roth, J. R. (1996) *J. Bacteriol.* **178,** 2825–2835.
7. Krasnow, M. A. & Cozzarelli, N. R. (1983) *Cell* **32,** 1313–1324.
8. Boocock, M. R., Brown, J. S. & Sherratt, D. J. (1986) *Biochem. Soc. Trans.* **14,** 214–216.
9. Scheirer, K. & Higgins, N. P. (2001) *Biochimie* **83,** 155–159.
10. Lilley, D. M. J. (1992) *Methods Enzymol.* **212,** 133–139.
11. Pruss, G. (1985) *J. Mol. Biol.* **185,** 51–63.
12. Pruss, G. & Drlica, K. (1985) *J. Bacteriol.* **164,** 947–949.
13. Margolin, P., Zumstein, L., Sternglanz, R. & Wang, J. C. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 5437–5441.
14. Lodge, J. K., Kazic, T. & Berg, D. E. (1989) *J. Bacteriol.* **171,** 2181–2187.
15. Lynch, A. S. & Wang, J. C. (1993) *J. Bacteriol.* **175,** 1645–1655.
16. Bochner, B. R., Huang, G.-C., Schieven, G. L. & Ames, B. N. (1980) *J. Bacteriol.* **143,** 926–933.
17. Bliska, J. B., Benjamin, H. W. & Cozzarelli, N. R. (1991) *J. Biol. Chem.* **266,** 2041–2047.
18. Datsenko, K. A. & Wanner, B. L. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 6640–6645.
19. Staczek, P. & Higgins, N. P. (1998) *Mol. Microbiol.* **29,** 1435–1448.
20. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY).
21. Pruss, G. & Drlica, K. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 8952–8956.
22. Drolet, M., Broccoli, S., Rallu, F., Hraiky, C., Fortin, C., Masse, E. & Baaklini, I. (2003) *Front. Biosci.* **8,** D210–D221.
23. Figueroa, N. & Bossi, L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9416–9420.
24. Pruss, G. J. & Drlica, K. (1989) *Cell* **56,** 521–523.
25. Norris, V. (1995) *Mol. Microbiol.* **16,** 1051–1057.
26. Woldringh, C. L., Jensen, P. R. & Westerhoff, H. V. (1995) *FEMS Microbiol. Lett.* **131,** 235–242.
27. Berens, C., Pfleiderer, K., Helbl, V. & Hillen, W. (1995) *Mol. Microbiol.* **18,** 437–448.
28. Hinrichs, W., Kisker, C., Duvel, M., Muller, A., Tovar, K., Hillen, W. & Saenger, W. (1994) *Science* **264,** 418–420.
29. Manna, D. & Higgins, N. P. (1999) *Mol. Microbiol.* **32,** 595–606.
30. Keiler, K. C., Waller, P. R. H. & Sauer, R. T. (1996) *Science* **271,** 990–993.
31. Cook, D. N., Ma, D., Pon, N. G. & Hearst, J. E. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10603–10607.
32. Gamper, H. B. & Hearst, J. E. (1982) *Cell* **29,** 81–90.
33. Liu, L. F. & Wang, J. C. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7024–7027.
34. Wu, H.-Y., Shyy, S., Wang, J. C. & Liu, L. F. (1988) *Cell* **53,** 433–440.
35. Higgins, N. P. & Vologodskii, A., in *Plasmid Biology*, ed. Funnell, B. (Am. Soc. Microbiol., Washington, D.C.), in press.
36. French, S. (1992) *Science* **258,** 1362–1365.
37. Wei, Y., Lee, J.-M., Richmond, C., Blattner, F. R., Rafalski, J. A. & LaRossa, R. A. (2001) *J. Bacteriol.* **183,** 545–556.
38. Bernstein, J. A., Khodursky, A. B., Lin, P.-H., Lin-Chao, S. L. & Cohen, S. N. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 9697–9702.
39. Elowitz, M. B., Levine, A. J., Siggia, E. D. & Swain, P. S. (2002) *Science* **297,** 1183–1186.
40. Ozbudak, E. M., Thattai, M., Kurtser, I., Grossman, A. D. & van Oudenaarden, A. (2002) *Nat. Genet.* **31,** 69–73.
41. Siegele, D. A. & Hu, J. C. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 8168–8172.
42. Domingo, E., Martinez-Salas, E., Sobrino, F., de la Torre, J. C., Portela, A., Ortin, J., Lopez-Galindez, C., Perez-Brefia, P., Villanueva, N., Najera, R., *et al.* (1985) *Gene* **40,** 1–8.
43. Segall, A. M. & Roth, J. R. (1994) *Genetics* **136,** 27–39.
44. Miesel, L., Segall, A. & Roth, J. R. (1994) *Genetics* **137,** 919–932.
45. Takeuchi, Y., Horiuchi, T. & Kobayashi, T. (2003) *Genes Dev.* **17,** 1497–1506.
46. Huertas, P. & Aguilera, A. (2003) *Mol. Cell* **12,** 711–721.
47. Yu, D., Sawitzke, J. A., Ellis, H. & Court, D. L. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 7099–7104.